PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

Eric ADRIAENSSENS et al. Group Art Unit: 1642

Application No.: 10/530,568 Examiner: M. HALVORSON

Filed: April 7, 2005 Docket No.: 123439

For: METHOD FOR NGF ASSAY FOR IN VITRO DIAGNOSIS OF BREAST CANCER AND THERAPEUTIC USE

DECLARATION UNDER 37 C.F.R. §1.132

I, Genevieve Choquet-Kastylevsky, a citizen of France, hereby declare and state:

1. I have a degree in Medicine (Dermatology and Immunology) that was conferred upon me by Lyon and Paris Universities in 1996, a Ph.D. in Immunology (Lyon University, 2001), and Master's Degrees in Molecular Biology (Paris University, 1990) and Pharmacology (Lyon University, 1997).

2. I have been employed by bioMerieux since 2001 and I have had a total of 7 years of work and research experience in diagnostic assay technology.

3. I am a member of the European Association of Cancer Research, and of the French Proteomic Society (SFEAP).

4. My publications include the following works in this field: Alix-Panabieres et al., Immunol Methods., 2005; Canelle et al., Electrophoresis, 2006; Perronet et al., Proteomics, 2006; Caron et al., Mol. Cell Proteomics, 2007; and Morla et al., Electrophoresis (in press). My public appearances include speaking engagements on proteomics and cancer before attendees to international symposiums.

5. I am a named inventor in the above-captioned patent application.

6. I am employed by one of the co-assignees of the above-identified patent application. In the course of that professional relationship, I receive compensation from that co-assignee for my work. I am not being separately compensated for my work in connection with this Declaration.

7. I declare the following:

A change in the level of a protein secreted by cancerous tissue does not necessarily correlate to a similar change in the level of the secreted protein present in blood, bone marrow, milk, cerebrospinal fluid or urine. Accordingly, a change in the level of NGF in breast cancer tissue, as taught by Sakamoto, would not have automatically correlated to a similar change of the level of secreted NGF in the blood, bone marrow, milk, cerebrospinal fluid or urine. It was surprisingly found by my co-inventors and me that in the case of breast cancer, overexpression of NGF in breast cancer tissue correlates to an increase in the level of secreted NGF in the blood, bone marrow, milk, cerebrospinal fluid and urine that can be used in breast cancer diagnostic methods.

I. Experimentation 1 Shows No Correlation Between Protein Levels Present In Tissue And Secreted Protein Levels Present In Bodily Fluids, Such As Blood, For A Particular Protein, "Protein 1"

A. Immunohistochemical Analysis

Immunohistochemical ("IHC") staining was performed under my direction in the pathology lab of Dijon Hospital, France. Human tissue samples were taken from: 1) normal colonic mucosa, distant from the tumor; 2) benign colonic adenoma; and 3) colonic adenocarcinoma. The tissue samples were stained with a mouse monoclonal antibody specific for Protein 1 and an anti-mouse antibody labeled with peroxidase, and visualized with 3-amino-9-ethyl carbazole (AEC). See Figure 1. The identify of Protein 1 is not divulged for trade secret reasons. As shown in Figure 1, Protein 1 was strongly overexpressed in tumor tissue as compared to normal colonic mucosa.

B. Seric Analysis

Seric analysis was performed in the proteomic lab of bioMérieux R&D, under my direction. Serum was taken from 29 healthy individuals and 29 colorectal cancer patients. It was analyzed by ELISA by using a mouse antibody specific for Protein 1 as the capture protein, a biotinylated antibody as the detection protein, and was visualized with avidin-alkaline phosphatase and its substrate. Figure 2 shows quantification of seric levels of Protein 1. Figure 2(a) and Table 1 show the standardization curve for Protein 1, and the corresponding data, respectively, indicating that the ELISA assay was sensitive and robust. Table 2 shows the seric doses of Protein 1 in the sera of each of the 29 colorectal patients (CRC+) and the 29 healthy controls. (Decimals are shown in European format with "," rather than ".".) Figure 2(b) shows the seric dose repartition of the samples taken from colorectal patients and healthy controls. The mean seric concentration of Protein 1 in each group is indicated by a bar in Figure 2(b). The mean concentration of Protein 1 in the serum of healthy individuals was 14.5 ng/ml. The mean concentration of Protein 1 in the serum of colorectal cancer patients was 12.6 ng/ml.

Experimentation 1 shows that an increased level of a protein secreted by colorectal cancer tissue does not correlate to an increased level of the secreted protein in blood, even though the protein is secreted by the cancerous colorectal tissue (the secretion of Protein 1 by colorectal cancer cells was confirmed in an ELISPOT assay, data not shown). IHC analysis shows overexpression of Protein 1 in tissues of cancer patients, as well as tissues of patients having benign lesions, as compared to tissues of healthy subjects. However, the mean concentrations of Protein 1 in the blood of both cancer patients and healthy subjects are equivalent. This demonstrates that overexpression of a protein in cancer tissue does not necessarily lead to an increase in the secreted protein in the blood, even though the protein is secreted by the cancerous tissue. Consequently, Experimentation 1 demonstrates that

determining the presence in a biological sample of a protein secreted by cancerous tissue cannot be predicted to be useful for diagnosis of the cancer, wherein the biological sample comprises a substance selected from the group consisting of: blood, bone marrow, milk, cerebrospinal fluid and urine.

II. Experimentation 2 Shows a Correlation Between NGF Levels Present in Breast Cancer Tissue and Secreted NGF Levels Present in Bodily Fluids, Such as Blood

The claimed invention is demonstrated by the results of Experimentation 2, which shows that an increased level of NGF present in breast cancer tissue correlates with an increased level of secreted NGF in blood.

A. Immunohistochemical Analysis

IHC analysis was performed under Hubert Hondermarck's supervision, in his laboratory at Lille University. Human breast cancer (invasive ductal breast carcinoma) tissue samples were obtained from 10 patients treated by mastectomy, and normal human mammary tissue samples (noncancerous mammary gland biopsies provided from mammoplasty material) were obtained from 10 subjects. Staining was performed on formalin-fixed paraffin-embedded (FFPE) tissue, using a specific anti-NGF rabbit polyclonal antibody, a biotinylated anti-rabbit IgG antibody, and a streptavidin-horseradish peroxidase solution. Immunoreactivity was visualized with 3-3'-diaminobenzidine tetrahydrochloride (DAB). As shown in Figure 3, breast cancer tissues expressed NGF, while normal mammary tissues showed no NGF expression. These results are in accordance with the results of Sakamoto et al.

B. Seric Analysis

Seric analysis was performed in the laboratory of Hubert Hondermarck, under the combined supervision of Hubert Hondermonk and me. Seric analysis revealed that detectable levels of NGF were present in the serum of breast cancer patients. Proteins present in the serum of breast cancer patients were separated based on molecular weight via SDS

polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto a polyvinylidene fluoride (PVDF) membrane by electrophoretic transfer, as shown in Figure 4. Lanes 1 and 2 represent samples obtained from breast cancer patients 1 and 2, respectively. NGF (Molecular Weight ~13 KDa) was identified by staining the membrane with an anti-NGF antibody. NGF was not detectable in the sera from healthy subjects. These results show that NGF secreted by breast cancer tissue in the blood can be used as a diagnostic tool for breast cancer.

Figure 4 is the best copy available. For ease of visualization, the PVDF membrane depicted in Figure 4 is reproduced in Figure 5. In each lane, any band near MW ~13 KDa in the PVDF membrane representing NGF present in the serum has been circled.

III. Experimentation 1 and Experimentation 2 Produce Different Outcomes

The results of experimentation 1 show that it would not have been predictable that the secretion of a protein by cancerous tissue would have correlated to the presence of the secreted protein in bodily fluids, such as blood. Accordingly, it would not have been predictable that the secretion of NGF by breast cancer tissue would have correlated to elevated levels of secreted NGF present in samples such as blood, bone marrow, milk, cerebrospinal fluid and urine. However, my co-inventors and I have discovered that the secretion of NGF by breast cancer tissue correlates to elevated levels of secreted NGF present various samples such as blood, bone marrow, milk, cerebrospinal fluid and urine.

IV. The Teachings of Bigazzi are Inapplicable to the Claimed Invention

The teachings of Bigazzi are unrelated and inapplicable to the claimed invention for many reasons. First, Bigazzi's teachings are directed to thyroid cancer, and Bigazzi is silent as to breast cancer. One of ordinary skill in the art would have appreciated that thyroid cancer is histopathologically and physiologically different from breast cancer. Furthermore, Bigazzi merely speculates that the factor found in the serum of the patient was NGF, but does

not conclusively identify the factor as NGF. See page 105, describing that the patient's serum had "high levels of a factor, *probably circulating human nerve growth factor*" (emphasis added). See also page 108 ("the material identified [as being present in the serum] was not proved to be human NGF"). Moreover, Bigazzi was written in 1976. There were no assay methods available in 1976 that would have positively identified the factor as NGF. Additionally, the patient's son, who was apparently clinically normal (i.e., had no tumors that could produce and excrete a biomarker) had high levels of the unidentified factor in the serum. See page 105. In light of the son's complex hereditary pathology, this strongly indicates that the unidentified factor was released into the serum from a source other than tumor cells.

V. Sakamoto Does Not Teach or Suggest How Much, if Any, of the Secreted NGF is Present in Blood, Bone Marrow, Milk, Cerebrospinal Fluid and Urine

Sakamoto teaches that NGF is involved both a paracrine loop and an autocrine loop. See page 977. One of ordinary skill in the art would have known that paracrine and autocrine loops involve cell signaling mechanisms, and that according to Sakamoto's teaching, NGF acts as a signaling molecule in the paracrine and autocrine loops. Therefore, Sakamoto teaches that the secreted NGF acts as a signaling molecule by binding to NGF receptors p75NGFR and TrkA. However, Sakamoto fails to teach or suggest how much, if any, of the NGF secreted from the breast cancer tissue is present in blood, bone marrow, milk, cerebrospinal fluid and urine, instead of participating in the paracrine and autocrine loops. Therefore, without such information, it would have been unpredictable for the presence of secreted NGF to be detected in blood, bone marrow, milk, cerebrospinal fluid and urine merely because it is secreted by breast cancer tissue.

Additionally, as demonstrated by Tria et al., Tandrup et al., and Krewson et al., NGF has a very short half-life (varying from less than one hour to five hours according to the

experimental protocols).[1] The short half-life of NGF would have made it even more unpredictable for the presence of secreted NGF to be detected in blood, bone marrow, milk, cerebrospinal fluid and urine. Secreted NGF in the blood quickly binds to spinal nodes rich in TRKa receptors, thereby depleting the secreted NGF from the blood. This also would have made it even more unpredictable that NGF in blood, bone marrow, milk, cerebrospinal fluid and urine could be used as a diagnostic tool for breast cancer. In spite of all of these facts, my co-inventors and I have unexpectedly discovered that NGF secreted by breast cancer tissue in blood, bone marrow, milk, cerebrospinal fluid and urine can be used as a diagnostic tool for breast cancer.

VI. Validating and Screening a Known Diagnostic Tool For a Disease Involves Mere Routine Experimentation to One of Ordinary Skill in the Art

Identifying a diagnostic tool that can usefully be detected in samples such as blood, bone marrow, milk, cerebrospinal fluid and urine is a challenging task that requires more than mere routine experimentation by one of ordinary skill in the art. However, validating and screening a diagnostic tool that is known to be usefully detectable in samples such as blood, bone marrow, milk, cerebrospinal fluid and urine involves mere routine experimentation to one of ordinary skill in the art.

My co-inventors and I have identified that detecting NGF secreted by breast cancer cells in blood, bone marrow, milk, cerebrospinal fluid and urine can be used as a diagnostic tool for breast cancer and therefore bringing the method to successful clinical application would have required merely routine experimentation from one of ordinary skill in the art.

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[1] Tria MA, Fusco M, Vantini G, Mariot R., *Pharmacokinetics of Nerve Growth Factor (NGF) Following Different Routes of Administration to Adult Rats.* Exp. Neurol.,127(2):178-83 (1994).
Tandrup, T., Vestergaard, S., Tomlinson, D. R., Diemel, L. T., Jakobsen, J., *The structural effect of systemic NGF treatment on permanently axotomised dorsal root ganglion cells in adult rats.*, J. Anat., 194(3): 373-379, (Apr. 1999).
Krewson, C. E., Saltzman, W. M. *Transport and elimination of recombinant human NGF during long-term delivery to the brain*, Brain Research, 727(2): 169-181 (1996).

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and/or imprisonment under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Date: **07/22/2008**

Genevieve Choquet-Kastylevsky

Figure 1: IHC analysis of colorectal tissue. The presence of Protein 1 was detected by using a monoclonal antibody specific for Protein 1 and an anti-mouse antibody labeled with peroxidase, and visualized with AEC staining. Very slight staining is shown in normal mucosa (a); moderate staining is shown in adenoma (b); and strong staining is shown in adenocarcinoma (c).

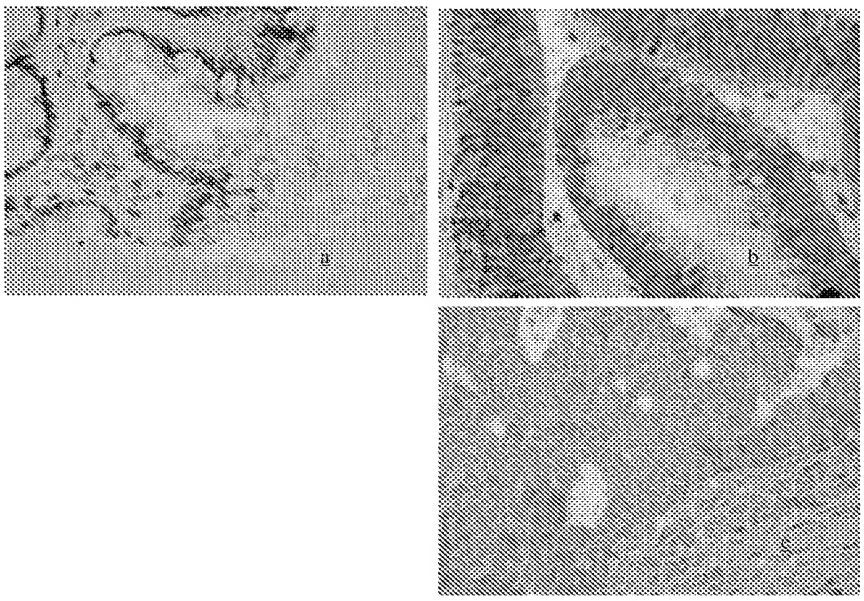

Figure 2: Quantification of seric levels of Protein 1. The mean concentration of Protein 1 is equivalent in the serum of colorectal cancer patients (CRC+) and healthy controls. Table 2 shows seric Protein 1 concentration of each of 29 colorectal cancer patients and 29 healthy controls.

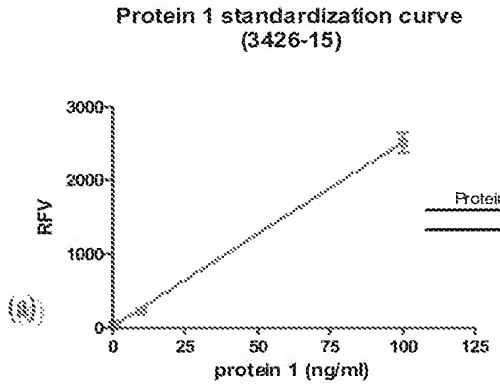

Table 1

Protein 1 ng/ml	RFV	
	Y1	Y2
100.000	2384.00	2649.00
10.000	231.00	236.00
1.000	58.00	50.00
0.000	23.00	26.00

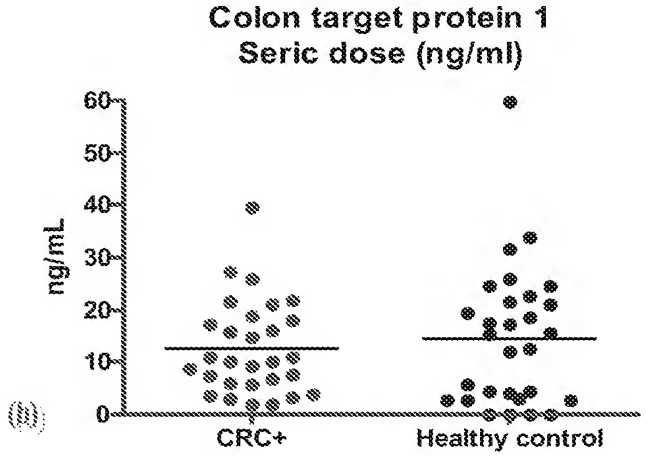

Table 2

Seric dose, ng/ml in colorectal cancer patients (CRC+) and healthy patients

CRC+	Healthy control
16,0348	31,6085
7,5021	17,217
7,2666	11,9808
10,88	25,7462
39,5144	15,3652
20,9644	18,5574
6,0111	15,2077
6,678	22,5045
9,3083	19,3463
9,9368	24,5201
10,88	21,4381
17,0199	12,5708
9,9761	17,414
8,6014	33,6717
27,1708	59,4989
18,6363	20,8459
15,7591	24,4015
21,3592	0
17,966	4,2468
25,944	0
21,6356	0,0178
1,7006	0
3,3063	4,0116
3,6198	2,7971
2,8754	4,2076
1,8181	2,5229
14,7746	2,5621
5,5013	2,9146
3,5806	5,6582

Figure 3: IHC analysis of breast tissue. The presence of NGF was detected by using an anti-NGF monoclonal antibody, and visualized with DAB. Normal mucosa (a) shows no staining, indicating the absence of NGF. In contrast, strong staining is seen in breast cancer tissue (b, c), indicating the presence of NGF, as described in Sakamoto et al.

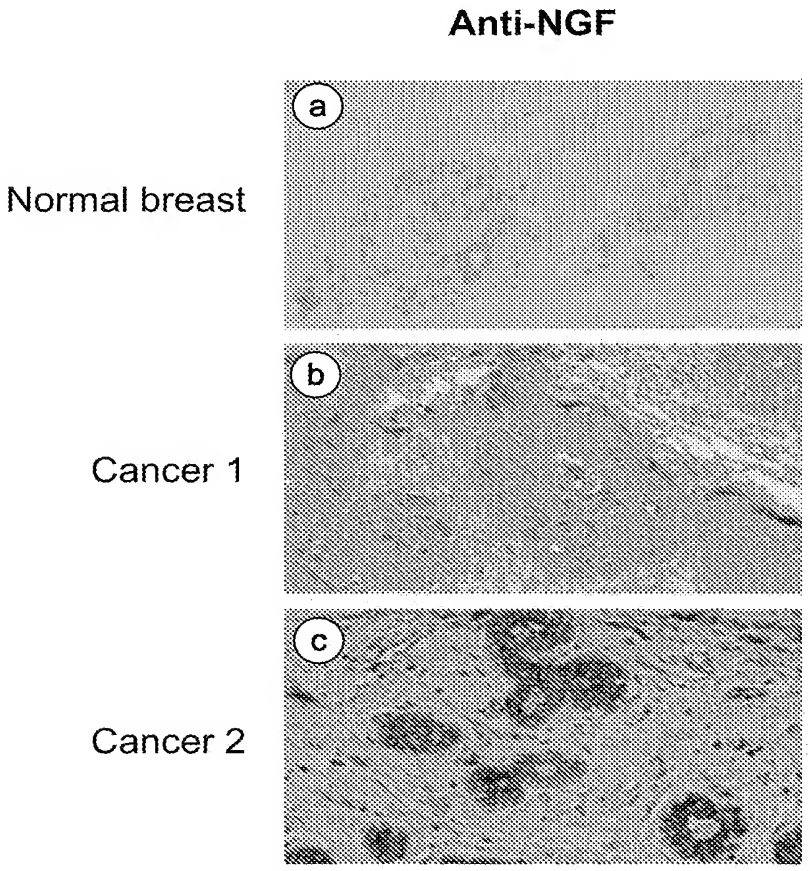

Figure 4: Detection of NGF in the serum of breast cancer patients (1, 2). A Western Blot and subsequent transfer onto a PVDF membrane. Lanes 1 and 2 represent samples obtained from breast cancer patients 1 and 2, respectively. NGF (MW ~13 KDa) was identified by using an anti-NGF antibody.

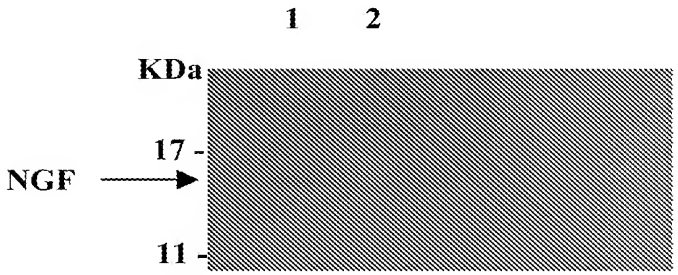

Figure 5: A reproduction of the PVDF membrane depicted in Figure 4. Lanes 1 and 2 represent samples obtained from breast cancer patients 1 and 2, respectively. In each lane, the band near (MW ~13 KDa) in the PVDF membrane representing NGF present in the serum has been circled for ease of visualization.